

Research paper

Clonality testing as complementary tool in the assessment of different patient groups with canine chronic enteropathy

Nicole Luckschander-Zeller^{a,*}, Sabine E. Hammer^b, Barbara C. Ruetgen^c, Alexander Tichy^d, Johann G. Thalhammer^a, E. Haas^a, Barbara Richter^e, Monika Welle^f, Iwan A. Burgener^a^a Clinic for Internal Medicine, Department of Companion Animals and Horses, University of Veterinary Medicine Vienna, Austria^b Institute of Immunology, Department of Pathobiology, University of Veterinary Medicine Vienna, Austria^c Clinical Pathology Platform, Department of Pathobiology, University of Veterinary Medicine Vienna, Austria^d Bioinformatics and Biostatistics Platform, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria^e Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine Vienna, Austria^f Institute for Animal Pathology, Vetsuisse Faculty, University of Bern, Bern, Switzerland

ARTICLE INFO

Keywords:

Canine chronic enteropathy
 Canine intestinal lymphoma
 Antigen receptor gene rearrangement
 Clonality testing
 Unspecific amplification

ABSTRACT

Differentiation between canine chronic enteropathy (CCE) and intestinal lymphoma is a diagnostic challenge as histopathology might fail to yield unequivocal results. Detection of clonal rearrangements of the T-cell-receptor gamma (TCRG) chain and IG heavy chain (IGH) V-J genes offer a useful solution. In this retrospective study, histopathology samples of 35 CCE patients and 7 healthy Beagle dogs underwent clonality testing. Patients suffered either from inflammatory bowel disease (IBD), food responsive diarrhea (FRD) or protein losing enteropathy secondary to IBD (PLE/IBD). Healthy Beagles served as controls (CO).

Canine IBD activity index (CIBDAI) and histopathological WSAVA-grading differed significantly ($p \leq 0.001$) between groups. CIBDAI improved significantly after appropriate therapy ($p < 0.0001$). Intestinal biopsies of all CO showed polyclonal patterns for B- and T-cell primers. All samples from CCE patients showed polyclonal patterns for the B-cell primers. Targeting TCRG, 4 patients showed a monoclonal or oligoclonal pattern of the lymphocytic infiltrates in the duodenum and/or colon. Clinical improvement was observed in all dogs. Although a small cell lymphoma cannot be excluded in view of the short follow up duration, a false positive result, in the sense of a canonical rearrangement or unspecific amplification due to an antigenic stimulation in a non-neoplastic inflammatory process is possible.

1. Introduction

Canine chronic enteropathy (CCE) is a common intestinal disease, which is characterized by chronic gastrointestinal signs, absence of underlying infectious, endocrinologic or neoplastic diseases and presence of inflammatory infiltrates in the intestinal walls verified by histopathology (Carrasco et al., 2015; Ohmi et al., 2017). CCE is categorized in different groups according to response to therapy; dogs suffering from food responsive diarrhea (FRD), antibiotic responsive diarrhea (ARD) and inflammatory bowel disease (IBD), which usually means that patients are glucocorticoid-responsive (Allenspach, 2013). Protein losing enteropathy (PLE) is a specific form of CCE and characterized by gastrointestinal protein loss, hypoalbuminemia and a worse prognosis compared to CCE without protein loss (Allenspach

et al., 2007). Dogs suffering from intestinal lymphomas generally present with identical or similar chronic gastrointestinal symptoms as dogs with CCE (Carrasco et al., 2015). Histopathology is the gold standard to distinguish inflammatory from neoplastic processes in the intestinal wall of endoscopic or surgical biopsies (Guilford et al., 1996), although sampling error or severe inflammation might preclude a correct diagnosis (Leibman et al., 2003). Additionally, immunohistochemistry stainings are used to improve the differentiation between chronic intestinal inflammatory processes and intestinal lymphoma (Burnett et al., 2003; Carrasco et al., 2015). Polymerase chain reaction for the antigen receptor gene rearrangement (PARR, conforming clonality testing) detects a clonal population of lymphocytes by amplification of T-cell receptor gamma-chain (TCRG) gene and immunoglobulin heavy chain gene (IGH) V-J gene rearrangements. Therefore, clonality testing

* Corresponding author at: Clinic for Internal Medicine, Department of Companion Animals and Horses, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210, Vienna, Austria.

E-mail address: Nicole.Luckschander@vetmeduni.ac.at (N. Luckschander-Zeller).

<https://doi.org/10.1016/j.vetimm.2019.109893>

Received 8 March 2019; Received in revised form 5 July 2019; Accepted 9 July 2019

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can be used as a complementary tool to differentiate inflammation from lymphoma. Nevertheless, in humans as well as in cats and dogs intestinal T-cell lymphoma can be considered a sequel of chronic irritation due to chronic inflammatory gastrointestinal diseases. It is hypothesized that a transgression from CCE to intestinal lymphoma, especially enteropathy-associated T-cell lymphoma (EATL) is a subtle process (Ferreri et al., 2011; Ohmi et al., 2017). On the other hand, mutations in the JAK/STAT pathway, suggesting a dysregulation in the cytokine pathway are described in the pathogenesis of EATL, emphasizing the genetic factors contributing to the diseases aetiology (Chander et al., 2018). Also in veterinary medicine genetic factors, environmental influence and infectious agents might contribute to the aetiology of intestinal lymphoma (Paulin et al., 2018; Willard, 2012). Hence, clonality assays might aid to establish a prognosis for CCE patients in order to predict the development of EATL, or to support the distinction between CCE and EATL. Furthermore, it might be important to see a clonal difference between different groups of CCE, in order to specify the prognosis of these patients. In this retrospective study, clonality testing of histopathology samples of 35 CCE-patients and 7 healthy Beagles was performed and differences of clonality patterns between IBD, FRD, PLE/IBD were investigated in consideration of clinical and histopathological grading as well as response to therapy.

2. Material and methods

2.1. Study population – sample origin

In total, 35 patient owned dogs suffering from CCE were included in this study, which was designed as a prospective investigation and characterization of FRD vs. IBD and their response to standard therapy (Burgener et al., 2008). Seven Beagles served as a control group. From this former study, remnant snap frozen intestinal biopsies from the CCE patients were retrospectively investigated and compared to formalin fixed tissue of Beagles including histopathological re-evaluation by WSAVA grading and clonality testing (2013–2014). Immunohistochemistry for CD3 and Paired box protein Pax5 was additionally performed on remaining samples from the four dogs with monoclonal results.

2.2. Clinical data of the study population

Owner consensus and study approval from the Cantonal Committee of Animal Experimentation, Bern, Switzerland (Nr. 118/05) was given.

The 35 dogs initially presented with chronic gastrointestinal signs for more than 6 weeks' duration. For clinical work up, the canine inflammatory bowel disease activity index (CIBDAI; Jergens et al., 2003) was determined and complete CBC, blood chemistry, fecal flotation, Giardia-Antigen test, ACTH stimulation test (except 2 dogs) and abdominal ultrasound were performed to exclude endocrinologic, infectious or neoplastic diseases. Animals did not receive antibiotic or immunosuppressive treatments at least 14 days before endoscopy. Duodenal and colonic samples were taken by endoscopy. The dogs were grouped according to clinical signs and response to treatment into FRD, IBD, and PLE/IBD. The response to treatment was defined as: FRD = improvement of CIBDAI in the first two weeks with elimination diet, IBD = no improvement with diet in the first 2 weeks, prednisolone treatment added. PLE/IBD group received from the beginning prednisolone and was named PLE/IBD group because inflammatory intestinal wall infiltration was seen in all dogs. After 2 weeks (1st recheck), 4 weeks (2nd recheck) and 10 weeks (3rd recheck), dogs were re-evaluated and a new CIBDAI determined.

2.3. Control animals – the Beagle cohort

Intestinal formalin fixed tissue of 7 Beagle dogs served as controls. Remnant material of these dogs was included retrospectively. Protocols

for this study were approved by the institutional Ethics Committee, the Advisory Committee for Animal Experiments (§12 of Law for Animal Experiments, Tierversuchsgesetz), and the Federal Ministry for Science and Research (referring number: GZ 68.205/0201-II/3b/2010). The Beagles were judged as healthy based on clinical examination (CIBDAI), CBC and blood chemistry results. Duodenal and colonic biopsies were taken by endoscopy.

2.4. Sample analysis by histopathology and immunohistochemistry

Biopsy samples (5–10/dog) of the 35 patients and the 7 control Beagle dogs were fixed in formalin, embedded in paraffin, processed, sectioned and stained with hematoxylin-eosin according to standard protocols. Histopathology of duodenal and colonic biopsies was assessed by one board certified pathologist blinded to the dogs' history and clinical exam using the WSAVA grading (Day et al., 2008). Furthermore, immunohistochemistry was performed on intestinal samples from four monoclonal CCE dogs using an autostainer 360 (Lab Vision, Thermo Fisher Scientific, Bonn, Germany). Specific antibodies for CD3 (polyclonal anti-rabbit, dilution 1:1.000, Dako, Vienna, Austria) and Paired box protein Pax 5 (monoclonal anti-mouse, dilution 1:100, Dako, Vienna, Austria) were used. The HRP-Polymer method was used. For CD3 and Pax 5 slides were pre-treated with heat in citrate buffer (pH 6) for 20 min for antigen unmasking. To decrease background staining slides were incubated in Hydrogen Peroxidase Block (Thermo Scientific) for five minutes and in Ultra Vision Protein Block (Thermo Scientific) for another ten minutes. A polyclonal rabbit anti-human antibody against CD3 (Dako, Vienna, Austria; diluted 1:1000) and a monoclonal anti-mouse antibody against Paired box protein Pax 5 (Dako, Vienna, Austria; diluted 1:100) were used. The samples were incubated with the primary antibodies for 30 min, subsequently by the secondary antibodies (Bright Vision poly HRP anti rabbit IgG and Bright Vision poly HRP anti mouse IgG both: Immunologic, Netherlands) for 30 min. For visualisation DAB Quanto (Thermo Scientific) for 5 min was used. Slides were counterstained with Mayer's haematoxylin, dehydrated, put into Neo Clear and mounted in Neo-Mount (both Merck, Darmstadt, Germany).

2.5. Clonality assays

For clonality testing, DNA extraction was performed on serial sections from snap frozen patient tissue as well as from formalin fixed tissue (Beagles) using a commercial kit following the manufacturers' instructions (GenElute™ Mammalian Genomic DNA Miniprep Kit; Sigma, Vienna, Austria). DNA quality check was performed using the NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) in pedestal mode. The minimum DNA concentration required was 30 ng/μl. As DNA purification check the 260/280 ratio was required to be around 1.8 (1.8–2.0). The PCR protocol for clonality testing was performed as previously described (Rütgen et al., 2016). In brief, each PCR reaction was carried out in triplicate, with positive and negative PCR controls included in each PCR run (Rütgen et al., 2016). The reactions were carried out using a T Gradient thermal cycler (Biometra, Göttingen, Germany) with amplification conditions for the IGH and TCRG primer sets as described (Valli et al., 2017; Keller and Moore, 2012). The PCR products were separated using the QIAxcel Advanced System capillary electrophoresis analyser with the QIAxcel DNA High Resolution Kit and the QX Alignment Marker 15 bp/1000 bp (Qiagen, Hilden, Germany). The presence and size of fragments was accurately determined using QIAxcel ScreenGel Software and the EuroClonality/BIOMED-2 guidelines for clonality testing in humans were applied for curve interpretation (Keller et al., 2016; Hammer et al., 2017).

2.6. Statistical evaluation

Statistical analyses were performed using the GraphPad Prism 7.0

Table 1
Descriptive statistics of CCE dogs and CO.

Dogs	Age years (M±SD)	Gender	Breed	Weight kg (M±SD)
FRD	3.3±3.0	3 male castrated 4 male 5 female spayed 6 female	2 mixed breed dogs, 2 French Bulldogs, 2 Labrador Retrievers, 1 Dachshound, 1 Canadian Shepherd dog, 1 Cairn Terrier, 1 German Shepherd, 1 Golden Retriever, 1 Newfoundland dog, 1 Pomeranian, 1 Samojed dog, 1 Tervueren, 1 Weimaraner, 1 West Highland White Terrier, 1 Yorkshire Terrier	22.3±15.3
IBD	5.0±3.8	4 male castrated 2 male 2 female spayed 1 female	3 Mixed breed dogs, 1 American Cocker, 1 Australian Shepherd dog, 1 Cavalier King Charles Spaniel, 1 Golden Retriever, 1 Malinois, 1 Sharpei	21.8±10.4
PLE/IBD	6.0±3.0	2 male castrated 1 male 4 female spayed 1 female	2 Pugs, 1 Buceron, 1 Bernese Mountain dog, 1 Labrador, 1 Mixed breed dog, 1 Rottweiler, 1 Yorkshire Terrier	19.1±12.1
CO	3.2±1.8	7 males	7 Beagles	17.6±1.5

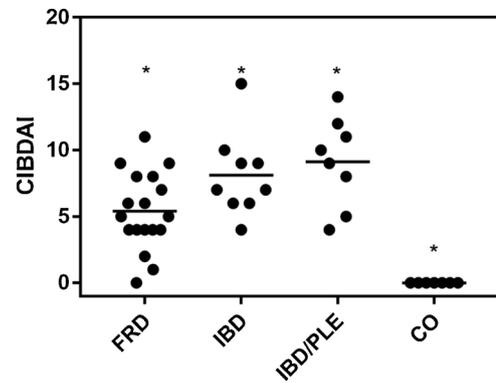


Fig. 1. CIBDAI of the different study groups. FRD (food responsive diarrhea; n = 18), IBD (inflammatory bowel disease; n = 9), PLE (protein losing enteropathy; n = 8), CO (control dogs, n = 7). Statistically significant differences (Kruskal-Wallis analysis) were detected between CO and all groups of CCE (p < 0.001). No statistically significant differences were detected between the CCE groups.

scientific statistic software (GraphPad Prism, GraphPad Software Inc., San Diego, California). Data were tested for normal distribution using the Shapiro Wilk-test. Since the normal distribution cannot be assumed for all groups, a Kruskal-Wallis analysis testing and a nonparametric Mann Whitney U test were used to evaluate the results. Statistical significance was set at p < 0.05.

3. Results

3.1. Descriptive statistics for the canine patients and control dogs

Biopsy samples of thirty-five dogs with chronic gastrointestinal signs and 7 control Beagle dogs could be retrospectively included in this study. The age did not differ between the groups. Breed, gender and weight in all groups were different (Table 1).

3.2. Canine IBD activity index (CIBDAI)

CIBDAI showed significant differences (Kruskal-Wallis analysis) between the CCE groups (FRD mean 5.4±2.9; IBD 8.1±3.2, IBD/PLE 9.1±3.4) and the control group (CO mean 0, p < 0.001; Fig. 1). The CIBDAI (Kruskal-Wallis analysis) improved significantly during the 10 weeks of therapy (mean 6.9±3.4 vs. 1.5±2.7; p < 0.0001; Fig. 2). Considering the different groups, the FRD (mean 5.4±2.9 vs. 0 + 0)

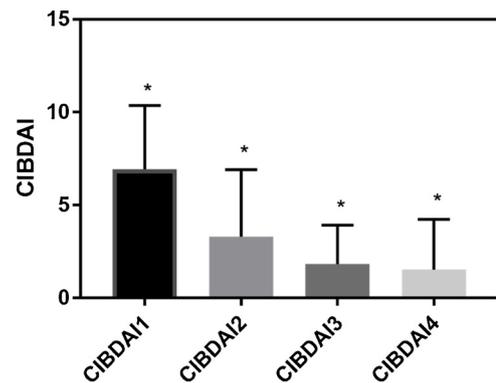


Fig. 2. CIBDAI (1–4) of all canine chronic enteropathy dogs (CCE) during therapy. CIBDAI1 = time of presentation, CIBDAI2 = 2 weeks after presentation, CIBDAI3 = 4 weeks after presentation, CIBDAI4 = 10 weeks after presentation (Kruskal -Wallis analysis). Note the significant decline of CIBDAI (Mann Whitney U test) during the study period (end 10 weeks after initial presentation) (p < 0.0001).

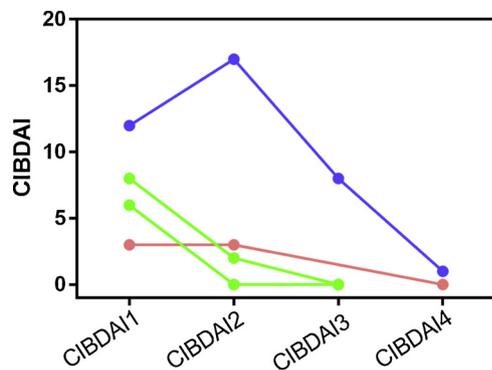


Fig. 3. Dot graph showing the improvement of the 4 dogs with mono/oligo-clonal result. The 2 dogs with the green lines belong to the FRD group, the pink line represents the IBD dog, as the blue line represents the IBD/PLE dog. Note the decline of CIBDAI in all dogs.

and the IBD (mean 8.1 + 3.2 vs. 2.1 + 1.7) improved significantly ($p < 0.0001$; $p = 0.0003$) (Mann Whitney U test) as the IBD/PLE (9.1 + 3.4 vs. 2.6 + 4.7) patients showed a trend of improvement but did not reach significant level ($p = 0.052$). All 4 dogs with mono/oligo-clonal results showed improvement in their CIBDAI score (Fig. 3).

3.3. WSAVA grading and immunohistochemistry

The histopathological grading of the duodenal samples in the small bowel was significantly different between the patient groups (Kruskal-Wallis analysis) (FRD mean 4.6 ± 1.9; IBD 4.9 ± 2.3; IBD/PLE 5.1 ± 1.8) and the control group (CO 1.3 ± 0.5; $p < 0.001$). There were no significant differences between the CCE groups (Fig. 4). All four patients with monoclonal results in the duodenal biopsy and one of the colon samples showed only a low grade mixed T-cell and plasmacytic population in the lamina propria and a low grade intraepithelial T-cell infiltration. Only in one dog Pax 5-positive B-cells were forming a small follicle in the lamina propria of one small intestinal sample. Neither T-nor B-cells exhibited atypia or an increased mitotic rate, and there was no morphologic evidence for a neoplastic process in any of the four dogs.

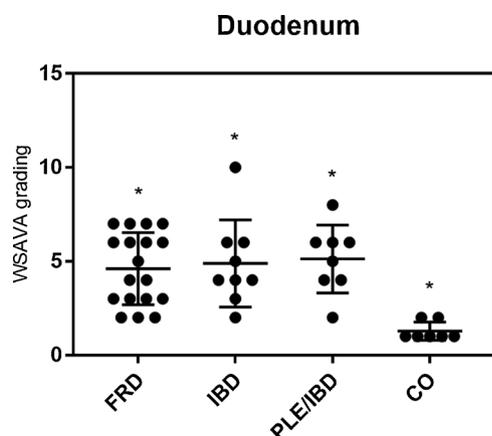


Fig. 4. WSAVA grading of duodenal biopsies in the different study groups. FRD (food responsive diarrhea; n = 18), IBD (inflammatory bowel disease; n = 9), PLE (protein losing enteropathy; n = 8), CO (control dogs, n = 7). Statistically significant differences were detected between CO and all CCE groups (Kruskal-Wallis analysis). No statistically significant differences were detected between the CCE groups. x-axis: different study groups, y-axis: the number of the patients in the different groups.

3.4. Clonality testing

The entire cohort of control Beagle dogs showed polyclonal electrophoretic patterns for the applied TCRG (T-cell clonality) and IGH (B-cell clonality) primer sets. Likewise, for the latter, all samples from the CCE patient group showed polyclonal patterns for the B-cell primer sets. Targeting TCRG, four patients showed mono- or oligoclonal patterns of the lymphocytic infiltrates (Fig. 5). Two of these dogs (monoclonal expression in the duodenum) belonged to the FR group, one dog with monoclonal expression in duodenum and oligoclonal expression in the colon to the IBD group, and one patient (oligoclonal expression in the colon) to the IBD/PLE group.

4. Discussion

This is the first study, in which different CCE groups were compared in regard to their distinct clonality patterns compared to young healthy Beagle dogs. Although we expected a different outcome between the groups, we could not detect any differences in clonality. In 4 of 35 CCE cases, mono- or oligoclonal patterns were detected in the TCRG primer sets. All affected patients showed significant improvement during appropriate therapy. The suspicion of more monoclonal expression in the PLE-dogs, as monoclonality is described as negative prognostic factor of PLE, was not found (Nakashima et al., 2015). None of the mono/oligo-clonal cases was diagnosed with lymphoma based on histopathology. These surprising results offer two possible explanations. First, clonality testing is a highly sensitive method so that latent intestinal lymphoma might be detectable even in endoscopic biopsies (Kaneko et al., 2009). Second, monoclonal rearrangement occurs also in inflammatory processes and thus clonal lymphocyte expansion can also be detected in benign CCE cases. In this study clonality testing was performed on snap frozen samples due to the retrospective nature of the study. This method is well described (Gress et al., 2016), but in spite of excellent DNA retrieval it cannot be completely excluded that this might have influenced incongruent results between histopathology, immunohistochemistry and clonality testing. However, in case that latent lymphoma would have been detected by clonality testing improvement without appropriate anti-tumour therapy would not be expected. In our case series positive clonality testing did not influence the outcome of the disease within the 10-week observation period. This could suggest that the second explanation is most likely, although the short observation period is one of the study limitation, and canine gastrointestinal small cell lymphoma might improve shortly on glucocorticoid therapy (Lane et al., 2018; Couto et al., 2018).

IBD in humans is regarded as a contributing factor in carcinogenesis and the development of EATL, arising from intestinal intraepithelial lymphocytes (IEL) (Ullman et al., 2011). Furthermore, EATLs are distinguished into EATL type I (common type, mostly associated with celiac disease) and EATL type II (Chan et al., 2011). EATL type II is mostly not associated with coeliac disease, and shows monomorphic T-lymphocytes by lacking the inflammatory infiltrates (Tan et al., 2013).

The development from IBD to EATL is also suggested in dogs (Evans et al., 2006). In dogs, gastrointestinal lymphomas are predominantly of T-cell origin (Matsumoto et al., 2018) and often associated with a lympho-plasmacytic inflammatory background (Miura et al., 2004). Subclassification of canine lymphoma in either EATL type I or type II is often not distinguished, but is already described (Valli et al., 2017). However, it is also suggested in the dog, that intestinal T-cell lymphomas develop from intraepithelial lymphocytes (Coyle et al., 2004), expressing CD3 (Matsumoto et al., 2018). Accordingly, we could only detect T-cell clonality in cell which were CD3 positive cells in immunohistology. In our study, all dogs, including the 4 dogs with mono- or oligoclonal TCRG patterns, improved significantly during therapy, suggesting clonal rearrangement in inflammatory lymphocytes. However, the time frame of 10 weeks might be too short to assess long term consequences of ongoing inflammation. There was no significant

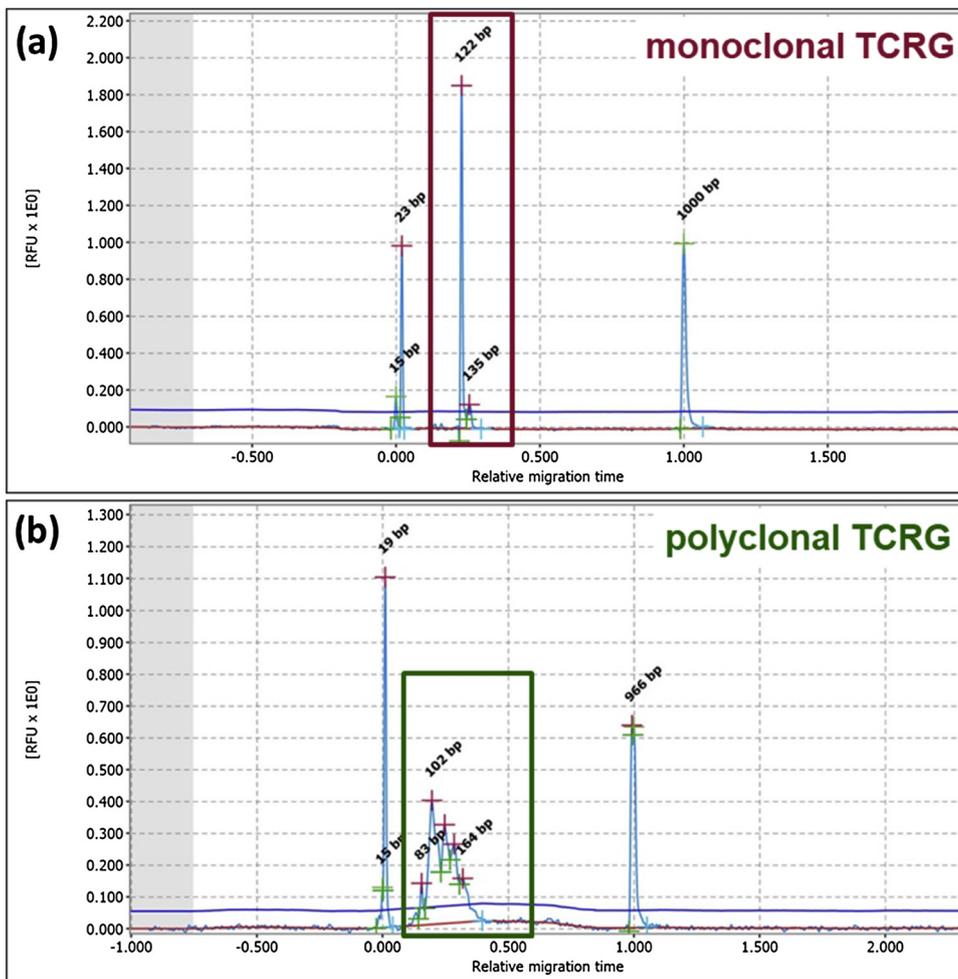


Fig. 5. Capillary electropherograms from clonality assays of the analysed duodenal samples are shown for two representative canine cases: (a) a CCE dog, showing a monoclonal result and (b) a representative example of a polyclonal pattern in the duodenum of one Beagle (CO). In the clonality assay, primer sets for T-cell (TCRG) clonality are shown and the result of clonality testing is displayed along with the raw signal. The peaks marked with green asterisks represent the alignment markers (15 and 1000 base-pairs). The presence and size of fragments was accurately determined using QIAxcel ScreenGel Software (Qiagen). RFU indicates relative fluorescence units.

difference in clonality detection between the CCE groups, although it is hypothesized that with exacerbation of inflammation, the chance for yielding clonal results might increase (Hiyoshi et al., 2015).

In human medicine, it is not uncommon to detect monoclonal expression in autoimmune and non-neoplastic diseases such as *Helicobacter pylori* gastritis, food allergy (Calvert et al., 1996) or cutaneous pseudolymphomas (Boer et al., 2008), features which are discussed as preliminary stages. The phenomenon of receiving a monoclonal result in a morphologically non-neoplastic population was also described in veterinary medicine such as regressing histiocytoma, hepatitis associated drug hypersensitivity reaction and infectious disease like Ehrlichiosis and Leishmaniosis. This was explained by canonical rearrangement and unspecific amplification (Keller et al., 2016). It is therefore mandatory to interpret clonality testing results always in the context of clinical signs, immunophenotyping and histopathological results. Furthermore an adequate follow up of the patient is mandatory since false positive clonality results in CCE patients are possible. Despite the possibility that clonal rearrangement might be found in inflammatory processes, data indicate that found clonality without any other sign of malignancy still warrants a conservative therapeutic approach. More research in terms of prolonged observation periods and routine application of clonality testing in the diagnostic workup of CCE is necessary to assess the diagnostic value of this versatile molecular approach.

Acknowledgements

The authors would like to thank Ilse Schwendenwein for critical review of this manuscript.

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